

# Suppression of Cell Motility and Metastasis by Transfection with Human Motility-related Protein (MRP-1/CD9) DNA

By Shuichi Ikeyama,\* Masaru Koyama,\* Masuo Yamaoko,\* Reiko Sasada,† and Masayuki Miyake§

From \*Department VI, Pharmaceutical Research Laboratories III, Pharmaceutical Research Division, †Department V, Discovery Research Laboratories II, Discovery Research Division, Takeda Chemical Industries, Ltd., Osaka 532; and the §Department of Thoracic Surgery, Kitano Hospital, Tazuke Kofukai Medical Research Institute, Osaka 530, Japan

## Summary

Previously we showed that motility-related protein (MRP-1) is an antigen recognized by monoclonal antibody (mAb) M31-15 inhibiting cell motility and that the sequence of MRP-1 coincides with that of CD9. In the present study, plasmid was constructed in which human MRP-1/CD9 cDNA is expressed under the control of the Abelson murine leukemia virus promoter sequence. The expression plasmid for MRP-1/CD9 was introduced into Chinese hamster ovary cells, human lung adenocarcinoma cell line MAC10 (MRP-1 positive), and human myeloma cell line ARH77 (MRP-1 negative). All of the MRP-1/CD9 (over)expressing clones obtained from these transfected cells showed suppressed cell motility (penetration and phagokinetic track assays) depending on the degree of expression of MRP-1/CD9. Overexpression of MRP-1/CD9 by MAC10 cells resulted in the suppression of cell motility (maximally 73%) associated with considerable inhibition of the cell growth (maximally 48%). However, the inhibition of the growth of MAC10 cells by mAb M31-15 was <17% at an antibody concentration of 1–5  $\mu\text{g}/\text{ml}$ , which inhibits cell motility by >90%. These results suggest that MRP-1/CD9 directly regulates cell motility and may also affect cell growth. Effects on metastasis by the expression of MRP-1/CD9 were investigated with mouse melanoma BL6 cells–BALB/c *nu/nu* mouse system. Metastatic potential of all transformants expressing MRP-1/CD9 was lower than that of parent BL6 cells.

The human leukocyte-associated antigen CD9 was discovered as a human B lymphocyte differentiation antigen, and it was initially considered to be specific for acute lymphoblastic leukemic cells; however, CD9 antigen has been found to be widely expressed on many nonhemopoietic tissues including smooth muscle, fibroblasts, and various cancers (1, 2). Indeed, it is strongly expressed on platelets, where it mediates platelet activation and aggregation upon binding with mAbs (3), but its essential role in nonhemopoietic cells has not yet been clarified. Recently, we obtained the mAb M31-15 inhibiting cell motility and cloned motility-related protein (MRP-1)<sup>1</sup> cDNA recognized by this mAb (4). The sequence of MRP-1 was quite similar to that of CD9 (5). Sequence analysis revealed that MRP-1 is highly similar to B cell surface antigen CD37 (6), melanoma-associated antigen ME491 (7), the target of the antiproliferative antibody TAPA-1 (8), the human tumor-associated antigen CO-029

(9), and Sm23 antigen of the trematode parasite *Schistosoma mansoni* (10). MRP-1 is a cell surface protein expressed at a molecular mass of 25 and 28 kD in most solid human tumor cell lines, except hepatocellular carcinomas, but not in hamster or mouse cell lines (4). The essential function of this family of unique transmembrane proteins is unknown, especially in relation to cancer. As the first approach to determine the function of MRP-1/CD9, the MRP-1/CD9 cDNA was introduced into several cell lines, and the properties of the stable transformants were analyzed.

## Materials and Methods

**Cells and Plasmids.** MAC10 (a human lung adenocarcinoma cell line) (4), Raji cells (a human Burkitt's lymphoma cell line), ARH77 (a human myeloma cell line) (11), and BL6 (a mouse melanoma cell line) (12) were cultured in RPMI 1640 supplemented with 10% FCS, 10 mM HEPES, and 100  $\mu\text{g}/\text{ml}$  of kanamycin (growth medium). Dihydrofolate reductase–negative (DHFR<sup>-</sup>) CHO cells (13) were cultured in Ham's F-12 instead of RPMI 1640. To construct pTB1442 (Fig. 1), the 1.1-kb EcoRI fragment containing the human MRP-1/CD9 cDNA was inserted into pTB1308, which was constructed

<sup>1</sup> Abbreviations used in this paper: DHFR, dihydrofolate reductase; MRP-1, motility-related protein.

from pTB399 (14) by removing the IL-2 gene, and then digested with Sal-I-HindIII. The fragment (2.7 kb) containing MRP-1/CD9 cDNA was ligated with the 4.1-kb Sal-I-HindIII fragment containing the DHFR gene from pTB348 (14). The control plasmid was constructed in the same manner without inserting MRP-1/CD9 into pTB1308. In some experiments, the 1.9-kb HindIII fragment containing the neomycin phosphotransferase gene prepared from the pneoER5 plasmid was inserted into pTB1442 and used (pTB1503).

**Transfection and Isolation of Stable Clones.** A confluent monolayer of DHFR<sup>-</sup> CHO cells in a 6-cm dish was transfected with pTB1442 DNA (10 µg) by the calcium phosphate coprecipitation procedure (15, 16). The cells were cultured in DME containing proline (35 mg/liter) and dialyzed FCS, and growing colonies were selected. ARH77, Raji, MAC10, and BL6 cells were transfected by the electroporation method (17) using a genepulser (Bio-Rad Laboratories, Richmond, CA). Briefly, the cell suspensions (5 × 10<sup>6</sup>/0.5 ml of RPMI 1640) were mixed with 20 µg of pTB1503 DNA for ARH77, Raji, and BL6 cells, or with 20 µg of pTB1442 and 2 µg of pRc/CMV (Invitrogen, San Diego, CA) DNA for MAC10 cells, cooled on ice for 5 min, and pulsed at 0.3 Kv/500 µF. The cells transfected with DNA were suspended at 2.5 × 10<sup>5</sup>/ml in the growth medium and seeded in microplates (100 µl/well). After incubating for 2 d, the medium was replaced with fresh medium containing 1 mg/ml of G418 (geneticin sulfate; Sigma Chemical Co., St. Louis, MO). After 2 or 3 wk, the cells growing at a single colony were selected.

**Determination of Cell Motility.** Cell motility was determined by two assay systems, cell penetration (18) and phagokinetic track (19), as described previously (4). In the former system, cell migration was determined by the modified method. Briefly, 600 µl of RPMI 1640, 0.1% FCS was poured in the lower compartment of a Transwell (pore size, 5-µm diameter; Costar, Cambridge, MA), and cell suspensions (5 × 10<sup>4</sup> cells/100 µl of RPMI 1640, 0.05% FCS) were placed in the upper chamber. After culturing at 37°C for 16 h, cells penetrating into the lower chamber were counted.

**Cell Adhesion.** Cell adhesion to fibronectin (human species; Seikagaku Corp., Tokyo, Japan) and laminin (mouse species; Seikagaku Corp.) was measured. The coating of plates with fibronectin and laminin (Maxisorp F96; Nunc, Roskilde, Denmark) was performed at 0.4 µg/well. Cells harvested with trypsin were washed once with RPMI 1640, 1% FCS, suspended at 4 × 10<sup>5</sup>/ml, and seeded in the fibronectin- or laminin-coated plates (100 µl/well). After incubating at 37°C for 4 h, nonadherent cells were removed by washing two times with 150 µl of PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4). Each well was filled with 100 µl of RPMI 1640, 1% FCS, then 20 µl of MTT (Sigma Chemical Co.) solution (5 mg/ml in PBS) was added to each well followed by incubation at 37°C for 4 h. 100 µl of SDS solution (10% SDS in 0.01 N HCl) was added to each well followed by incubation at 37°C for 24 h. The absorbance was then read at 590 nm. The assay was performed in quadruplicate.

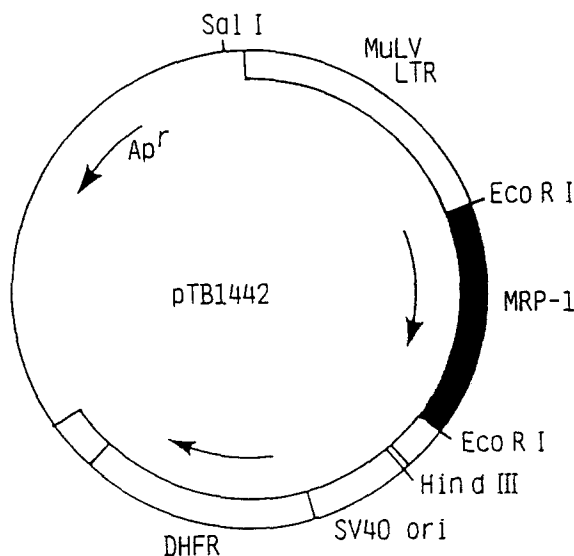
**Determination of MRP-1/CD9 Content.** MRP-1/CD9 content was determined by cytofluorometry (20) and cell ELISA. Cell ELISA was performed as follows. Cells were harvested with 0.01% EDTA and washed once with growth medium. Aliquots of 10<sup>5</sup> cells were placed in tubes, the medium was removed by aspiration, and 100 µl of medium A (RPMI 1640 containing 1% FCS and 0.1% NaN<sub>3</sub>) or mAb M31-15 (2 µg/ml in medium A) was added to each tube. After incubation at 0°C for 30 min, the cells were washed four times with 200 µl of RPMI 1640, 0.1% NaN<sub>3</sub>. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (100 µl; diluted 1,000-fold with medium A) was then added to each tube. After incubation at 0°C for 30 min, these cells were washed four times

with 200 µl of PBS. Freshly prepared substrate solution (200 µg of *o*-phenylenediamine and 0.05 µl of H<sub>2</sub>O<sub>2</sub> in 100 µl of 24.3 mM citric acid, 51.4 mM Na<sub>2</sub>HPO<sub>4</sub> [pH 5.0]) was added to each tube followed by incubation at room temperature for 10 min. The reaction was stopped by adding 200 µl of 1 N H<sub>2</sub>SO<sub>4</sub>, and aliquots of the supernatant were transferred to a 96-well plate. The absorbance at 490 nm of each well was read by a microplate reader (450; Bio-Rad Laboratories). The concentration of MRP-1/CD9 was expressed as relative concentration, where the concentration of MAC10 cells was determined tentatively as 1 U.

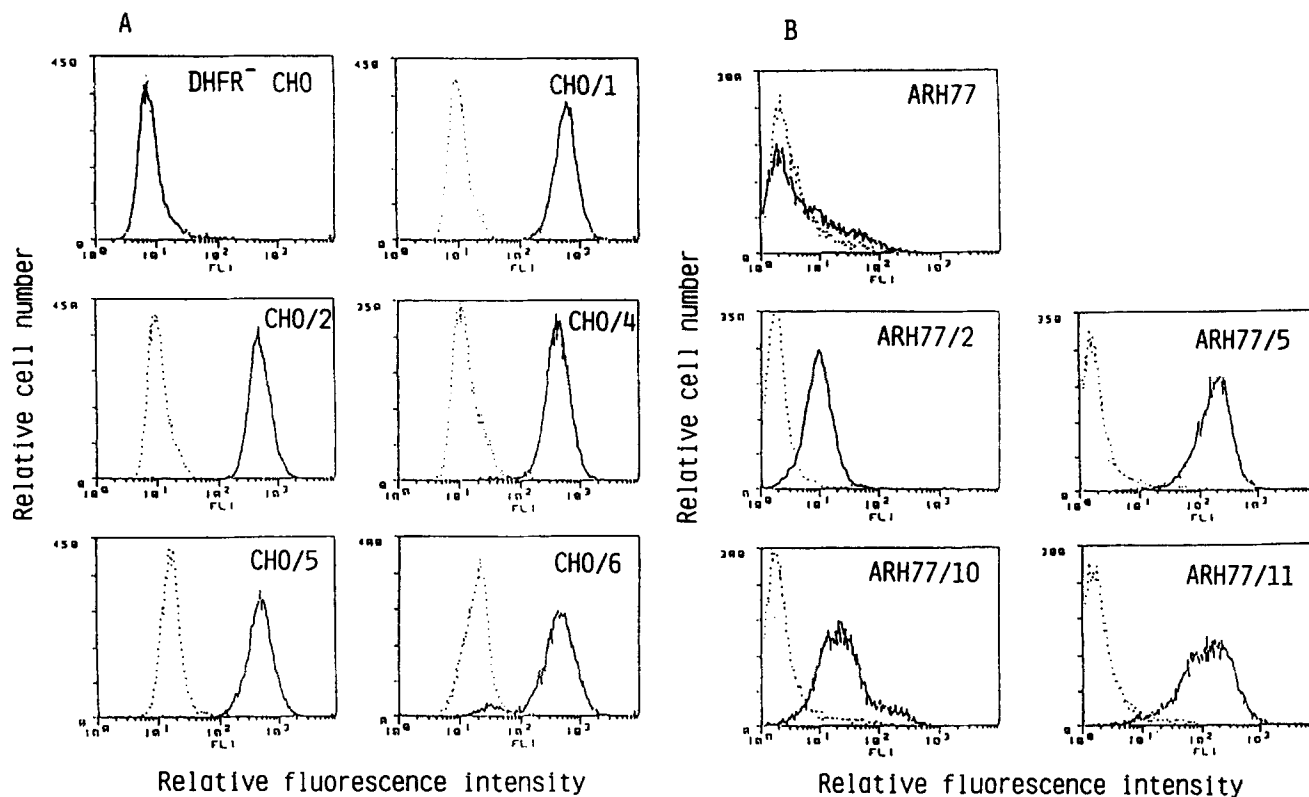
**Experimental Metastasis Assay.** Pulmonary metastasis was induced by intravenous challenge of BALB/c *nu/nu* mice (*n* = 10) with 10<sup>5</sup> cells. The number of pulmonary metastases was determined 14 d after challenge (18).

## Results

**Transfection and Isolation of Stable Clones.** DHFR<sup>-</sup> CHO cells were transfected with pTB1442 DNA, and growing colonies (DHFR<sup>+</sup>) were selected. The five clones obtained were tested for the expression of MRP-1/CD9 by cytofluorometry using mAb M31-15. All clones strongly expressed MRP-1/CD9 (Fig. 2 A). ARH77 and Raji cells were transfected with pTB1503 DNA and selected based upon resistance to G418. 10 clones were obtained for each, and four clones for ARH77 cells (Fig. 2 B) and five clones for Raji cells (data not shown) clearly expressed MRP-1/CD9. MAC10 cells were cotransfected with pTB1442 and pRc/CMV DNA and selected based upon resistance to G418. 14 clones were obtained and examined by cytofluorometry, but the degree of expression of MRP-1/CD9 was not clear (data not shown). Then, the MRP-1/CD9 content on the surface of MAC10 and its clones was determined by cell ELISA. Some clones expressed MRP-1/CD9 more strongly than the parent cells. Seven clones expressing MRP-1/CD9 in different degrees were selected for



**Figure 1.** Map of the expression vector pTB1442. The MRP-1 cDNA (MRP-1), the origin of replication (SV40 ori), dihydrofolate reductase (DHFR), ampicillin resistance gene (Apr), and the region of MuLV long terminal repeat (LTR) are indicated.



**Figure 2.** MRP-1/CD9 expression on various cells as defined by mAb M31-15. The expression was determined by cytofluorometric analysis. (A) DHFR<sup>-</sup> CHO and its clones; (B) ARH77 and its clones.

further detailed analysis (Table 1). MAC10 cells were also cotransfected with control (lacking MRP-1/CD9) DNA of pTB1442 and pRc/CMV DNA, and when colonies were selected based on G418 resistance, 12 clones were obtained.

The changes in MRP-1 content were not significant (data not shown).

**Motility of Various Clones.** Motility of the various clones was mainly determined by a cell penetration assay. Cell mo-

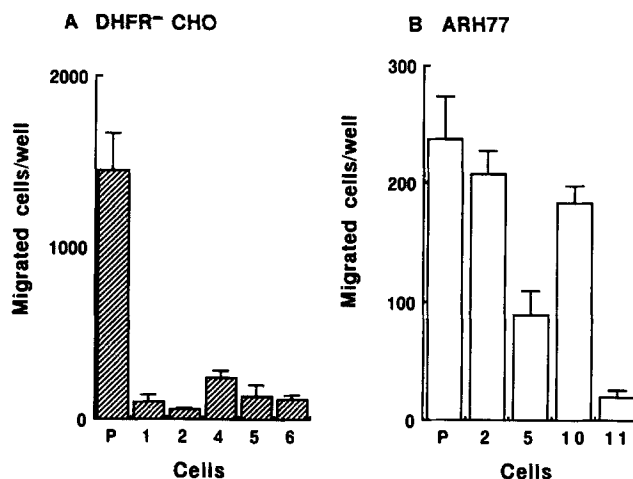
**Table 1.** MRP-1/CD9 Contents, Motility, and Cell Growth of Transformants and Their Parent MAC10 Cells

Cell	Relative MRP-1	Relative motility*	Relative growth
	<i>U</i>	%	%
MAC10	1.0	100	100
MAC10/1	1.20 ± 0.12	42	82 ± 9 <sup>†</sup>
MAC10/2	1.10 ± 0 <sup>‡</sup>	92	66 ± 10 <sup>‡</sup>
MAC10/5	1.10 ± 0.11	89	76 ± 14
MAC10/10	1.20 ± 0.1 <sup>‡</sup>	51	82 ± 16
MAC10/13	1.63 ± 0.1	28	63 ± 4
MAC10/19	1.48 ± 0.25	27	62 ± 12
MAC10/20	1.15 ± 0.06	65	71 ± 17

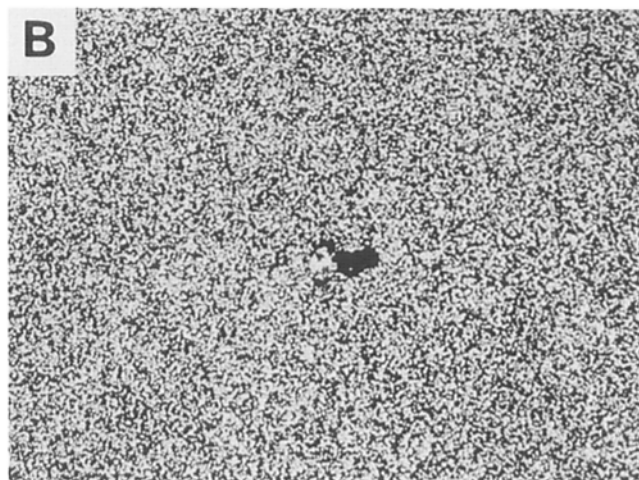
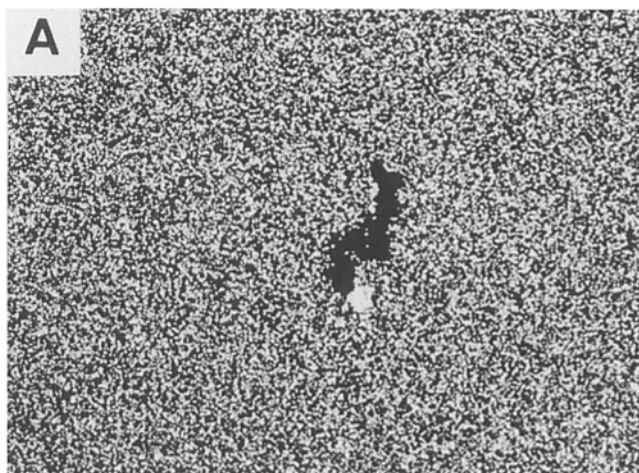
Values were determined as described in Materials and Methods. Data are calculated from four independent experiments, and each value represents the mean ± SD.

\* Average of two independent experiments.

† Average of three independent experiments.

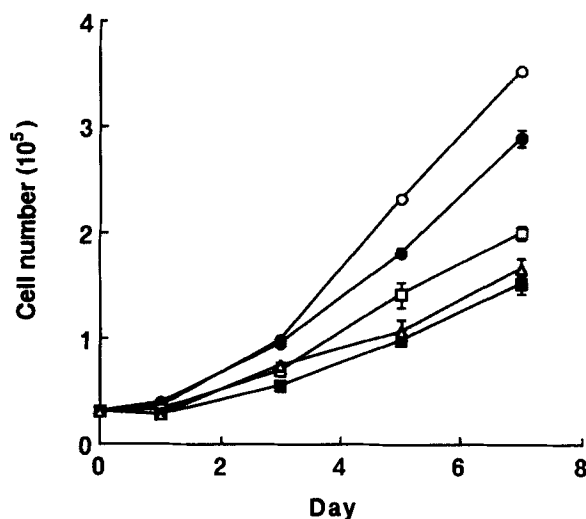


**Figure 3.** The motility of various cells as determined by the cell penetration assay. 100  $\mu$ l RPMI 1640, 0.1% FCS was placed in the lower Transwell component. The cells ( $5 \times 10^4$  for DHFR<sup>-</sup> CHO or  $2.5 \times 10^4$  for ARH77) were suspended in 100  $\mu$ l of RPMI 1640, 0.05% FCS, placed in the upper chamber, and cultured for 16 h. Cells penetrating into the lower chamber were then counted. (A) DHFR<sup>-</sup> CHO (P) and its clones (1-6, clone numbers); (B) ARH77 (P) and its clones (2-11, clone numbers). Each value represents the mean ± SD (bar) ( $n = 3$ ).



**Figure 4.** The motility of various cells as determined by phagokinesis in the gold colloid method.  $2 \times 10^3$  cells harvested with trypsin were plated on gold particle-coated coverslips in a 3.5-cm dish and left in the incubator for 16 h. Phagokinetic tracks were visualized under a darkfield microscope. (A) MAC10; (B) MAC10/13.

tility was strongly suppressed in the clones of DHFR<sup>-</sup> CHO (Fig. 3 A) and also suppressed in the ARH77 clones dependent on the degree of MRP-1/CD9 content (Figs. 2 B and 3 B). The motility of the clones derived from MAC10 cells was also suppressed dependent on the degree of the MRP-1/CD9 content (Table 1). The motility of the clones MAC10/13 and MAC10/19 was strongly suppressed (~75%). The 12 clones obtained by transfection with control DNA (lacking MRP-1/CD9) showed considerably different motility, that is, four clones had markedly high motility and four clones had low motility (data not shown). The average of the relative motility of the 12 clones was  $1.22 \pm 0.66$  (mean  $\pm$  SD) times that of the parent MAC10 cells. On the other hand, the average of the 14 clones obtained by transfection with DNA containing MRP-1/CD9 was  $0.60 \pm 0.23$  (mean  $\pm$  SD;  $p < 0.005$ ) times that of parent MAC10 cells (data not shown). This strongly supports the assumption that the expression of MRP-1/CD9 suppresses cell mo-

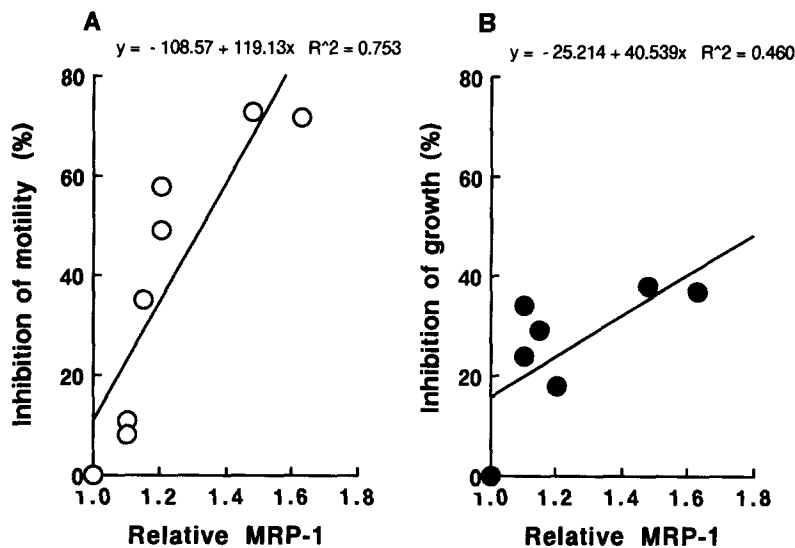


**Figure 5.** The growth curve for various cells.  $3 \times 10^4$  cells were seeded in each well (multidish 24; Nunc), and the plates were incubated at 37°C for 7 d. The cells on the plate were harvested with 0.125% trypsin, 0.01% EDTA, and the cell number was counted (Coulter Electronics Inc., Hialeah, FL). MAC10 (○); MAC10/2 (●); MAC10/3 (□); MAC10/13 (■); MAC10/19 (△). Each value represents the mean  $\pm$  SD (bar) ( $n = 3$ ).

tility. For Raji cells, parent Raji cells did not migrate (<10 cells/well) and no significant changes in the motility of clones were observed (data not shown). The suppression of motility was confirmed by a phagokinetic track assay using the clones derived from MAC10 cells. As shown in Fig. 4, strong suppression was confirmed. The average area ( $n = 50$ ) of MAC10 and MAC10/13 cells was  $8,366 \pm 3,250$  (mean  $\pm$  SD) and  $2,042 \pm 1,101 \mu\text{m}^2$ , respectively ( $p < 0.001$ ).

**Cell Adhesion.** The adhesion of cells to fibronectin and laminin was investigated. An incubation time of 4 h was chosen, since adhesion of the cells to the plates was weak and slow, especially in the case of fibronectin-coated plates (data not shown). MAC10/20 cells showed slightly strong adhesion to both fibronectin- and laminin-coated plates; however, no other clones showed any significant differences compared to parental MAC10 cells (data not shown).

**Cell Growth.** The growth of MAC10 and its clones was investigated. The inhibition of the growth of MAC10 cells by mAb M31-15 was weak and ~15% at 1–5  $\mu\text{g}/\text{ml}$  (data not shown). However, the inhibition in the clones obtained from MAC10 cells by transfection with MRP-1/CD9 DNA was considerably strong (Fig. 5). The inhibition of the cell growth seemed to be due to the elongation of the doubling time, since the inhibition was weak on day 1 compared with days 3, 5, or 7, and the degree of the inhibition was increased (Fig. 5). The inhibition on day 5 is summarized in Table 1. The growth of all clones was suppressed and the suppression for MAC10/2, MAC10/13, and MAC10/19 was ~35%. The average of the relative growth of the seven clones (Table 1) was 0.73 times that of the parent MAC10 cells. On the other hand, the average of the 12 clones obtained by transfection with control DNA was 1.10 times that of parent MAC10 cells (data not shown).



**Figure 6.** The correlation between MRP-1/CD9 content and the inhibition of cell motility and inhibition of cell growth. The data from Table 1 are plotted. (A) MRP-1/CD9 content vs. inhibition of cell motility. (B) MRP-1/CD9 content vs. inhibition of cell growth.

**Correlation of MRP-1/CD9, Cell Motility, and Cell Growth.** For MAC10 and its clones, the inhibition of the motility was plotted against the MRP-1/CD9 content (Fig. 6 A), and a relatively good correlation between them was found. The cell motility was markedly suppressed with overexpression of MRP-1/CD9. The inhibition of cell growth was also observed in all clones, but the correlation between MRP-1/CD9 content and the degree of growth inhibition was weak (Fig. 6 B).

**Metastatic Potential of BL6 and Its Transformant.** To determine the effect of metastasis by expressing MRP-1/CD9, several human cell lines containing MAC10 cells were tested for their ability to make metastatic foci in lung. However, we could not find a high and reproducible metastatic cell line. Thus, the ability of MRP-1/CD9 was evaluated by the use of high metastatic mouse melanoma BL6 cell line. BL6 cells were transfected with pTB1503 DNA and selected based upon resistance to G418 and we obtained four stable MRP-1/CD9-positive clones (seven G418 resistants) from two independent experiments. Cell motility and metastatic potential of all clones were examined (Table 2). Both cell motility and metastatic potential were suppressed in all clones expressing MRP-1/CD9.

**Table 2.** Metastatic Potential and Phagokinetic Motility of BL6 and Its Transformants

Cells	No. of metastatic foci in lung	Mean $\pm$ SD	Motility
			$\mu\text{m}^2 \pm \text{SD}$
BL6	327, 226, 218, 191, 261, 203, 127, 193, 187, 230	$216 \pm 52$	$8,663 \pm 3,228$
BL6/1	60, 44, 58, 83, 49, 85, 86, 44, 64, 41	$61 \pm 18^*$	$5,535 \pm 2,399^*$
BL6/2	45, 38, 53, 58, 56, 9, 66, 46, 39, 24	$43 \pm 17^*$	$5,523 \pm 2,288^*$
BL6/3	59, 71, 57, 83, 60, 72, 26, 81, 75, 19	$60 \pm 22^*$	$6,077 \pm 2,328^*$
BL6/6	42, 126, 91, 77, 77, 60, 62, 86, 78, 20	$72 \pm 29^*$	$4,440 \pm 1,885^*$

$10^5$  cells of high-metastatic mouse melanoma BL6 (MRP-1/CD9 negative) and its four clones (expressing MRP-1/CD9) were injected intravenously into BALB/c *nu/nu* mice. Metastatic foci in lungs were examined after 14 d. Motility was determined by phagokinetic tract assay as described in Materials and Methods.

\*  $p < 0.001$ .

## Discussion

Cell motility is a highly complex process dependent on pericellular adhesion molecules such as fibronectin and laminin, integrin receptors, cytoskeletal components, and a junctional unit concerning cytoskeletal components and membrane receptors (21, 22). A large variety of growth factors and autocrine factors have been shown to stimulate cell motility (23), which is an essential cellular function and plays a very important role in tumor invasion and metastasis, and metastasis is among the most important problems in the therapy of the patients with various kinds of cancers. The metastatic dissemination of tumor cells to secondary distinct sites requires highly motile behavior, so the regulation of tumor cell motility could lead to the suppression of metastasis.

MRP-1 was identified as motility-related protein using mAb M31-15 inhibiting the cell motility (4). The functions of MRP-1/CD9 were first investigated by the DNA transfections reported in this paper. The transfections were made with four different cell lines, DHFR<sup>-</sup> CHO (hamster; high motility), ARH77 (not expressing MRP-1/CD9; nonadherent; high motility), Raji (not expressing MRP-1/CD9; nonadherent; low motility), and MAC10 (expressing MRP-1/CD9 and used

for the motility assay in our previous paper [4]). Several stable transformant clones were obtained from these cell lines and analyzed for cell motility, cell growth, and cell adhesion to fibronectin and laminin. In MAC10 cells, MRP-1/CD9 content was determined by cell ELISA. The motility was strongly suppressed by the (over)expression of MRP-1/CD9 in all highly motile cell lines, such as hamster (DHFR<sup>-</sup> CHO) and MRP-1/CD9-negative (ARH77) and -positive (MAC10) human cell lines. In addition, the degree of motility suppression was dependent on the content of MRP-1/CD9 in the clones derived from both ARH77 (Figs. 2 B and 3 B) and MAC10 cells (Table 1 and Fig. 6 A). In the cell line with low motility (Raji), no significant changes were found (data not shown). These results strongly suggested that MRP-1/CD9 is not a motility-stimulating receptor for the motility factors existing in connective tissues or sera, and that it regulates cell motility by an unknown mechanism. The possibility remains that MRP-1/CD9 is a receptor for the negative signal ligands, but no evidence suggesting this possibility has been obtained at present, and this is the subject of further investigation.

The clones from MAC10 cells also showed strongly inhibited cell growth compared with the parent cells (Fig. 5 and Table 1), but the correlation between the MRP-1/CD9 content and the cell growth was weak (Fig. 6 B). Therefore, the inhibition of cell growth by transfecting MAC10 cells with MRP-1/CD9 DNA may be a secondary effect, since mAb M31-15 strongly inhibited cell motility (4) but not cell growth. However, the possibility of a direct effect cannot be ruled out, since TAPA-1 (8), a member of the same family, strongly inhibits the growth of some cells. Thus, the possibility that MRP-1/CD9 regulates both cell motility and cell growth remains.

Sequence analysis revealed that MRP-1 (4) shows high similarity with a new family of transmembrane proteins: CD37 (6), ME491 (7), TAPA-1 (8), CO-029 (9), and Sm23 (10). The functions of these proteins are not clear and no studies dealing with cell motility have been done. In this paper, we clearly showed that MRP-1/CD9 suppressed cell motility (Table 1 and Figs. 3, 4, and 6 A). It would be very interesting to know whether other proteins in this transmembrane family, such as ME491 or CO-029 expressed on solid human tumor cell lines, affect cell motility or not. The function of ME491 and CO-029 is unknown, but recently, Weterman et al. (24) reported that pMW4 (ME491) was expressed highly in less metastatic cell lines. This finding is consistent with our observation, that is, the expression of MRP-1/CD9-suppressed cell motility (Figs. 3, 4, and 6).

MRP-1/CD9 was expressed on almost all human solid tumor cell lines and weakly expressed or not expressed on human normal cells (4). It is very interesting that MRP-1/CD9 is expressed on cancer cells, since MRP-1/CD9 suppresses cell motility and cell growth, and this should be undesirable for cancer cells. Recently, Mohler et al. (25) reported that the existence of cancer cell motility-inhibitory protein in the serum-free medium conditioned by the nonmotile, non-metastatic cells. Therefore, cell motility may be regulated by both positive and negative signal ligands, and cancer cells may have their receptors. It is expected that controlling the expression of MRP-1/CD9 could lead to suppression of metastasis. Our preliminary experiment (Table 2) showed that metastatic potential of mouse melanoma cells was considerably suppressed by the expression of human MRP-1/CD9. We are planning to determine whether or not metastasis is also suppressed by the transfection of metastatic human cells with MRP-1/CD9 DNA.

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Address correspondence to Shuichi Ikeyama, Research and Developmental Division, Biology Research Laboratories, Takeda Chemical Industries, Ltd., 2-17-85 Jusohomachi, Yodogawa-ku, Osaka 532, Japan.

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